FEEDING EXPERIMENTS ON THE SUBSTITUTION OF PROTEIN BY DEFINITE MIXTURES OF ISOLATED AMINO ACIDS

BY

HAROLD HANSON MITCHELL

A. B., University of Illinois, 1909

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IN CHEMISTRY

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FEEDING EXPERIMENTS ON THE SUBSTITUTION OF PROTEIN BY DEFINITE MIXTURES OF ISOLATED AMINO-ACIDS.[1]

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Investigations concerned with the question of the indispensability of amino-acids to the animal organism and with their precise significance in metabolism, have been pursued notably along two general lines. The first line of investigation, although followed by several workers independently with varying results, has been perfected by Abderhalden and has given the most fruitful results in his hands. The method is based upon the fact, which has been repeatedly proven experimentally, that the animal organism can be maintained in equilibrium and can even exhibit considerable increases in weight and nitrogen when the nitrogenous requirements are covered by preparations obtained by hydrolyzing protein material with enzymes until no positive biuret test can be secured, when presumably only amino-acids and probably the simpler polypeptides were present. One of the most successful experiments of this nature was recently reported by Abderhalden.[2] In this experiment a dog was kept for 100 days on a hydrolyzed meat preparation (ereptone) supplemented by non-nitrogenous nutrients. During this time the animal behaved normally, seemed in perfect health, and increased in weight from 13.75 to 23.10 kg. Similar successful experiments in which completely

[1] The results presented in this paper formed part of a thesis submitted by the author to the Graduate School of the University of Illinois in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry.

[2] Abderhalden, E., *Z. physiol. Chem.*, 1913, lxxxiii, 444.

hydrolyzed preparations[3] were used during some of the periods have been still more recently reported by the same investigator,[4] using dogs and rats as experimental subjects.

On the basis of such results the essence of Abderhalden's method was to remove certain amino-acids from the unknown mixture of the products of protein hydrolysis as completely as available methods would permit, and to determine the nutritive value of the residual mixture of amino-acids by testing its ability to maintain body weight and nitrogen equilibrium when fed to experimental animals in an otherwise adequate ration as the sole source of nitrogen. If the residual mixture proved itself of equal nutritive value to the original, the conclusion seemed justified that the amino-acid or amino-acids removed were not indispensable to the body, but could be synthesized from other compounds, provided, of course, the removal was reasonably complete. If the residual mixture of amino-acids was clearly inadequate to cover the nitrogen requirements, the proof of the indispensability of the amino-acid or amino-acids removed was completed by adding again to the ration the substances removed (isolated in a pure condition) and thus restoring its complete nutritive value. In cases in which the mixture was still inadequate after such addition, no conclusions would be justified. Such ambiguous results might be produced, for example, by the removal of substances other than those intentionally removed, by the incomplete removal of reagents, such as mercury salts, which may exert a harmful effect on the animal organism, or by chemical changes in the residual amino-acids incidental to the removal of those under investigation.

In Abderhalden's laboratory this method has been applied with striking success in many instances. The inability of the body to synthesize tryptophane and tyrosine has been repeatedly and clearly shown. In these experiments the tryptophane was removed according to the method of Hopkins and Cole with mercuric sulfate in 5 per cent sulfuric acid solution, and the tyrosine

[3] The completeness of hydrolysis in these cases was determined by amino nitrogen determinations before and after boiling with concentrated mineral acid. These preparations were submitted first to the prolonged action of gastric, pancreatic, and intestinal juices or extracts and were then digested for 8 to 10 hours on the water bath with dilute sulfuric acid.

[4] Abderhalden, *Z. physiol. Chem.*, 1915, xcvi, 1.

by concentration of the neutralized solution *in vacuo*. The addition of tryptophane or tyrosine, respectively, to the residual mixtures in most experiments had a distinct effect in correcting their nutritive deficiencies entirely, as indicated by the maintenance of body weight of the experimental animals to which the mixtures were fed, and the restoration of a positive balance of nitrogen. Furthermore, in his more recent experiments Abderhalden has shown that tyrosine cannot be substituted by the corresponding keto acid, oxyphenyl pyruvic acid, or by phenyl pyruvic acid, nor could it be substituted satisfactorily in most experiments by phenylalanine, in accordance with the work of Embden and Baldes.[5] Some improvement, however, in nitrogen equilibrium was noted upon the addition of phenylalanine to the tyrosine-free ration, suggesting possibly a partial oxidation of this amino-acid to tyrosine.

The work on the nutritive value of tryptophane and tyrosine reviewed above embodies the only entirely successful results obtained with the method. Attempts to determine the nutritive value of proline and cystine have not led to results sufficiently clear cut to warrant final conclusions, while still less encouraging are the experiments to test the diamino-acids, by removal according to the procedure of Kossel and Kutscher.

Henriques and Hansen[6] have published the results of an experiment similar in character to the work of Abderhalden, which led them to conclude that the diamino-acids could be entirely dispensed with by the animal body. The experiment was performed on a rat and the ration contained, besides non-nitrogenous constituents, a mixture of the products of protein hydrolysis containing presumably only monoamino-acids. It was prepared from an enzyme digest of a protein by precipitation with phosphotungstic acid, filtration, removal of excess reagent from the filtrate, and evaporation to dryness *in vacuo*. Unfortunately the particular protein used was not mentioned, and the lack of all details of the phosphotungstic acid precipitation precludes any attempt to judge of the completeness of separation of monoamino- from diamino-acids. The experiment lasted only 26 days. During the last 17 days only were positive nitrogen balances obtained and they

[5] Embden, G., and Baldes, K., *Biochem. Z.*, 1913, lv, 301.

[6] Henriques, V., and Hansen, C., *Z. physiol. Chem.*, 1904–05, xliii, 417.

were such that they could hardly be said to constitute convincing evidence of the nutritive adequacy of the ration fed, especially in view of the fact[7] that the determination of the total urinary nitrogen per day of rats, with the exercise of the utmost care as to collection and preservation, is subject to errors of 10 per cent or more, due to incomplete collection. The body weight increased slightly during the last half of the experiment, but during the last 3 days it declined slightly but consistently. It is unfortunate that the experiment was not continued further to determine whether this final decrease was significant or not. This criticism is especially justified by the many experiments that may be quoted on the feeding of synthetic rations, in which entirely erroneous conclusions may be drawn if attention is confined to the first 20 or 30 days of observation.

The second general method of investigation of the nutritive significance of the amino-acids to the animal organism is associated more especially with the names of Osborne and Mendel and their associates, and has given by far the most valuable results in their hands. The method consists essentially in the feeding of isolated and carefully purified proteins, obtained from a wide variety of sources, incorporated in basal rations otherwise adequate for normal growth or maintenance and composed of artificial food materials, in most part of known composition and containing at most but minimal percentages of nitrogen or protein. By this method different proteins have been clearly shown to have different values in nutrition, in one respect or another, and these differences have been traced with great success to differences in amino-acid content. Thus, the significance of some of the amino-acids has been made evident, either by noting the peculiar effect attendant upon their absence in proteins or their occurrence in minute amounts, or by observing the effects produced upon supplementing proteins by their addition.

Of the results obtained by Osborne and Mendel with this second method of investigation the most general biological significance is attached to the demonstration that lysine is essential for growth. Gliadin, the alcohol-soluble protein of wheat, when fed

[7] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication No. 156*, 1911, pt. i, 12.

to rats as the sole protein in a ration, is capable of maintaining an animal for long periods of time at constant weight, but is not able to induce normal growth. A slow growth would often result, while at other times only maintenance was secured. This characteristic stunting with gliadin resulted even when the calorie intake was sufficient for optimum growth, and has been successfully traced[8] to the low content of this protein in lysine, since upon the addition of lysine to the ration, normal growth could be secured.

Zein, the alcohol-soluble protein of the corn kernel, has been shown by Osborne and Mendel (and others before them) to be unable to support either growth or maintenance. Upon analysis, this protein has been shown to lack the amino-acids glycocoll, tryptophane, and lysine. The addition of tryptophane to the protein renders it adequate for long continued maintenance,[9] the further addition of lysine renders it adequate for fairly normal growth,[9] but for normal growth a further addition of arginine, which is present only in small amount in zein, appears to be necessary.[10]

Recently, in an investigation[11] to determine the effect of different percentages of various proteins in the ration on the growth of white rats, Osborne and Mendel have shown that rations containing percentages of casein inadequate for normal growth may be rendered adequate by the addition of the sulfur-containing amino-acid cystine to the ration. These results are intelligible in view of the comparatively low cystine content of casein.[12]

In properly appraising the two general lines of investigation of the nutritive value of amino-acids briefly described—on the one hand, that method associated mainly with the name of Abderhalden, and, on the other hand, that exemplified best by the work of Osborne and Mendel—it must be granted that they have contrib-

[8] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 332.

[9] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 378.

[10] Osborne and Mendel, *J. Biol. Chem.*, 1914, xviii, 11.

[11] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351.

[12] Whether the favorable influence of cystine when added to rations containing 9 per cent or less of casein was due to its sulfur content entirely and could be duplicated by other available sulfur-bearing compounds is open to question. It is to be noted that the ration as a whole, as well as the casein, was poor in sulfur.

uted much valuable information. Our conceptions of protein metabolism and protein requirements must be profoundly modified as a result of this pioneer work. The work of Osborne and Mendel, in particular, is worthy of the highest commendation. By working with small experimental animals and using artificially prepared rations, consisting in large part of carefully purified substances of definite composition, they have succeeded in performing numerous experiments under carefully controlled conditions. The results have been clear cut and unequivocal in most instances. However, no criticism of these methods would be complete without due account being taken of their limitations.

The limitations of the Abderhalden method are obvious. It is only applicable to those amino-acids that can be readily and quantitatively isolated (or practically so) from protein hydrolysates, and isolated by means of reagents that can subsequently be entirely removed from the residual mixture. Furthermore it is essential to the success of the method that the residual amino-acids are not changed or destroyed during the manipulations involved in the removal of the amino-acid or acids to be tested, nor by the reagents employed for this purpose. In view of the difficulty, if not the impossibility, of conforming to the above conditions in the removal of the large majority of the amino-acids from mixtures obtained from the hydrolysis of proteins, this method is seriously restricted in its usefulness. It seems probable, in view of the methods available for the quantitative separation of amino-acids, that the Abderhalden procedure cannot be of any lasting value without at the same time becoming so cumbersome as to be of little practical utility.

The limitations of the Osborne and Mendel method are also obvious. Its success depends upon a knowledge of the amino-acid content of the different proteins investigated. The extremely unsatisfactory condition of this knowledge as regards the large majority of the proteins studied needs no comment. The most striking results have been afforded by a study of the nutritive value of proteins totally or largely deficient in one or more amino-acids. But such proteins are few in number, apparently, and their characteristic effects may involve some peculiar proportions among those amino-acids that do occur in them, proportions which cannot be intelligently modified in the absence of reliable data as

to their precise nature. A lack of such data, in the case of phaseolin for example,[13] has blocked any attempt to determine the cause of its inadequacy for either maintenance or growth. A similar situation exists in connection with the poor nutritive value for growth of many of the proteins of the leguminous seeds, such as vignin.[14]

Any intelligent supplementing of proteins with isolated amino-acids is also impossible in the absence of at least fairly reliable information of their amino-acid content. Thus Osborne and Mendel state[15] that they did not attempt to supplement lactalbumin with purified amino-acids to determine whether normal growth could be secured with less than 9 per cent of this protein in the ration, on account of inadequate information as to its amino-acid make-up.

However, even though a good knowledge of the amino-acid content of proteins were available, due to the evolution of accurate analytical methods, a study of the nutritive value of native proteins and of native proteins supplemented by amino-acids can hardly be expected to reveal the nutritive values of each individual amino-acid, nor to determine whether each amino-acid may or may not be synthesized by the animal organism. Much less could such a method of study be expected to yield complete information as to quantitative amino-acid requirements. The impossibility of such attainments by the use of the Osborne and Mendel method rests in the impossibility of controlling the amino-acid intake absolutely and of experimenting with any desired combination of amino-acids. For example, the suggestive theory of Osborne and Mendel[16] to the effect that the only reason for the destruction of any protein at all in maintenance might be to liberate a small amount of one or two, or at most a relatively small number of amino-acids, to engage in some "hormone-like physiological duty upon which proper metabolism might depend," obviously could not be tested by their method of experimentation.

Thus while it would be hazardous to attempt to predict to what extent scientific ingenuity will overcome the obstacles inherently

[13] Osborne and Mendel, *J. Biol. Chem.*, 1914, xviii, 9.
[14] Osborne and Mendel, *Z. physiol. Chem.*, 1912, lxxx, 316.
[15] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 356.
[16] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 328 and 329.

limiting the applicability of this method to the problems of amino-acid synthesis in the animal organism and of amino-acid requirements, it may be confidently asserted that only a very incomplete solution can be expected from even the most extensive experimentation with it.

The most direct method of attacking these problems, and the only method with unlimited possibilities, is the feeding of definite mixtures of isolated amino-acids, to the complete exclusion of proteins and all of their intermediary degradation products. It is the main purpose of this investigation to test the feasibility of this method of experimentation.

Several attempts to cover the nitrogenous requirements of dogs by substituting the protein of a ration by a mixture of purified amino-acids have been made by Abderhalden.[17] After many unsuccessful trials one experiment of 8 days' duration is reported in which the most encouraging results were obtained. The difficulties encountered in the previous trials were, first, inducing the animals to consume the rations containing amino-acids, and, second, avoiding the digestive disturbances which such rations were especially liable to produce, such as vomiting and diarrhea. In the 8 day experiment above mentioned a mixture of isolated amino-acids in their naturally occurring forms, containing all of the amino-acids commonly obtained in the hydrolysis of proteins with the exception of oxyproline, was fed. Each day's ration consisted of 25 gm. of amino-acids, 50 gm. of a glycerol-fatty acid mixture, 2 gm. of cholesterol, 50 gm. of glucose, and 5 gm. of bone ash, the amino-acids constituting about 9 per cent of the total ration. Though the ration possessed no decided taste, it was a matter of great difficulty to induce the animal to consume it. It was necessary to feed the dog often with small portions of the food. During the 8 days of feeding the body weight was constant. A negative nitrogen balance was obtained on the 4th, 5th, 6th, and 8th days, though the average balance of the period was positive. During the subsequent 7 day period, when 25 gm. of hydrolyzed beef (with a smaller percentage of nitrogen) were substituted for the amino-acid mixture in the above ration, the body weight increased slightly and a much more favorable nitrogen balance was secured.

Another dog was fed for 6 days with a ration containing 22.1 gm. of the same amino-acid mixture, 2 gm. of hydrolyzed nucleic acid, 25 gm. of fat, 20 gm. of sucrose, 20 gm. of glucose, and 5 gm. of bone ash. The weight of the dog was practically constant throughout the period, though negative nitrogen balances were obtained on the 3rd, 4th, and 5th days of feeding. The experiment was terminated by the refusal of the dog to consume any more of the ration. Substitution of the amino-acid mixture by hydrolyzed meat in the above ration was followed by more favorable results, as in the preceding experiment.

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[17] Abderhalden, *Z. physiol. Chem.*, 1912, lxxvii, 22.

Abderhalden concludes from these two feeding periods that no physiologically indispensable amino-acid was absent from the mixture fed. This conclusion, however, must be considerably discounted by reason of the shortness of the periods of amino-acid feeding, and by the unsatisfactory nitrogen balances obtained. The objections inherent in such short feeding periods as occur throughout Abderhalden's investigations need no comment in view of the frequent experience, especially of American investigators, with rations that appear to be adequate for long periods of time, but that ultimately lead to nutritive failure. Quoting a significant remark in one of the papers of Osborne and Mendel:[18]

"If we had been content to discontinue the experiments after a reasonable period, many of the declines [in body weight] evidently associated with imperfections in the dietary and readily checked by a change in feeding, would have escaped attention."

Osborne and Mendel[19] have recently reported briefly an attempt to cover the maintenance nitrogenous requirements of rats by a mixture of tryptophane, cystine, histidine, tyrosine, phenylalanine, proline, and ammonium citrate or urea. In their own words:

"All such attempts failed, even when the supply of energy in the form of non-protein substances was liberal and the food contained all of the necessary inorganic salts and 'food accessories,' and in addition at least 0.5 per cent of protein, present in the 'protein-free' milk. On such diets the rats declined just as rapidly as when the amino-acid additions were not made."

It is evident from this review of the few experiments concerned with the feeding of mixtures of amino-acids to the exclusion of

[18] Osborne and Mendel, *J. Biol. Chem.*, 1912–13, xiii, 245 and 246. Further evidence of the unreliability of short feeding experiments is afforded by the work of Dr. Ruth Wheeler. According to this investigator (*J. Exp. Zool.*, 1913, xv, 210):

"Animals have a surprising power of getting along for a time on a qualitatively inadequate diet. Mice kept upon a ration in which two-thirds of the protein was gelatin and the other third casein did not show a decline in body weight for 21 days, although the ultimate decline of every animal fed upon this food showed it to be insufficient; another group made material gains in weight for 17 days on a diet upon which all ultimately lost weight rapidly and died unless the food was changed."

[19] Osborne and Mendel, *J. Biol. Chem.*, 1916, xxv, 2.

protein that no conclusive results have thus far been obtained; that is, that no definite mixture of isolated amino-acids has been shown to be capable of supporting life indefinitely. With the purpose of discovering some such mixture, the experimental work reported below was undertaken. The results secured to date have not been entirely successful, but several points of interest have been observed that seemed of sufficient importance to warrant publication.

EXPERIMENTAL INVESTIGATION.

The Selection of Experimental Animals.—The arguments in favor of the use of the smaller experimental animals in nutrition work have been well summarized by Osborne and Mendel:[20]

"The preparation of suitable pure food supplies on a scale sufficient for long periods and in economically procurable amounts is thereby rendered possible. The necessary scientific measurements and analyses can be conducted on a scale impossible for larger animals, and the problem of care and attention is equally simplified. Experiments can be duplicated without great effort and individual peculiarities eliminated by force of numbers. Furthermore, the various stages of growth and maturity are completed in the smaller animals within relatively short periods of time, so that the permanent effects of any dietary become apparent within a range of days or months that is not outside of ordinary experimental possibilities of observation. As illustrations of some of these features it may be recalled that the ultimate effects of complete inanition are apparent in 4 or 5 days[21] in rats or mice; in dogs the fatal outcome may be delayed for many weeks. The duration of life in the white rat is about 3 years; about 280 days suffice to complete the entire period of growth to maturity."

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[20] Osborne and Mendel, *Carnegie Institution of Washington, Publication No. 156*, 1911, pt i, 6.

[21] In the course of one of our feeding experiments with white mice we attempted to feed an amino-acid ration to a female mouse weighing 23.6 gm. For the first 4 days the food was untouched; on the 5th day, 1.5 gm. of ration were eaten. For the next 2 days the food was untouched and it was withdrawn on the 7th day, from which time the animal received water only. The body weight rapidly declined, though aside from emaciation no other symptoms of malnutrition, such as roughened coat, infected eyes, etc., were apparent. On the 15th day of observation, *i.e.*, 10 days after the 1.5 gm. portion of food was eaten, the animal died at a weight of 11.0 gm., having lost over 53 per cent of its initial weight. From this observation it would appear that the resistance of mice to inanition is much greater than is indicated in the quotation cited.

In the experimental project reported below the main limiting factor was the amounts of amino-acids that could be bought or prepared. It was, therefore, considered expedient to use mice or rats for the work, and of these two species of animals mice were considered more satisfactory, since they are only about one-tenth as heavy as rats of equal age and consequently they require a much smaller amount of food for maintenance and growth. Their span of life is also shorter than that of the rat. It has been stated that the average life of a white mouse is less than 2 years, that they are sexually mature at 2 months of age, and are fully grown at 150 days or even less. It would therefore appear that the effect of any inadequacy in a ration would be manifested in an even shorter period of time with mice than with rats.

It is certainly of some importance to consider the suitability of mice (or rats) as experimental subjects, not only from the standpoint of experimental convenience and expediency, but also from the standpoint of the biological significance of any experimental results obtained. It is of no economic importance or of no human interest simply to determine the qualitative or quantitative food requirements of such animals as rats or mice, if such requirements are not indicative, in a general way at least, of the food requirements of all mammalian organisms.

One is apt to have the impression, probably largely on account of the great discrepancy in size between rats and mice and animals of any economic or other importance to man (including man himself), that these smaller experimental animals are inherently unsuited for investigation purposes, because any results obtained with them must possess a very limited significance. In opposition to such an impression stands the work of Folin and Morris[22] on the great similarity in the composition of the urine and also in purine metabolism between the rat and man. The extensive investigations of Donaldson[23] on the growth of the white rat have revealed close similarities to man, the similarity extending even to the growth of the brain and spinal cord. The close parallelism between the results that Hart and McCollum[24] have obtained

[22] Folin, O., and Morris, J. L., *J. Biol. Chem.*, 1913, xiv, 509.

[23] Donaldson, H. H., Boas Memorial Volume, New York, 1906, 5–26; also *J. Comp. Neurol. and Psychol.*, 1908, xviii, 345.

[24] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373.

with pigs and with white rats in their study of the influence of restricted rations on growth also testifes to the general biological significance of experimental results secured with rats and presumably, also, of those secured with mice.

Such concrete illustrations of the close biological relations existing between the different species of mammals lend support to the general practice among physiologists and investigators in nutrition and related fields—a practice that is perhaps too generally accepted and too seldom submitted to critical scrutiny—to attach a general biological significance to experimental results obtained upon one particular species, or more truly upon a few representatives of one particular species of animals. The main justification of this practice is that, though no extensive systematic investigations on the comparative physiology and metabolism of the different species of mammals have been attempted, the experimental findings upon a large variety of animals are all remarkably consistent with one another, if not actually confirmatory of one another. Those differences that have been found in the metabolism of different species of mammals are largely quantitative and not qualitative. Therefore it has been concluded that experimental results obtained with one species of mammals may be considered as at least qualitatively applicable to mammals in general. Obviously accurate observations on rats or mice should be considered as valuable, in their general applicability, as observations on any other species of animal.

Experimental Details.—The mice were confined in galvanized iron cages, 8 inches in diameter and 6 inches high. In most of the experiments two mice were confined in a single cage. A small amount of paper excelsior[25] was always kept in the cages.

The rations were placed in small porcelain crucibles, $1\frac{3}{4}$ inches in diameter at the top and about $1\frac{1}{8}$ inches in height, which were wired to the sides of the cages. To prevent undue scattering of the food, the top of the crucible was fitted with a flanged ring of tinned copper, the hole in the ring being about 1 inch in diameter. This was crossed by two wires, also of tinned copper, at right angles to each other, so that there were four holes through which the mice could insert only their heads. With this device it was

[25] The paper excelsior used in all of the work analyzed 0.80 per cent ash and 0.05 to 0.06 per cent nitrogen.

hoped that all scattering of the rations would be avoided, but almost invariably with the amino-acid rations considerable scattering would occur in spite of all precautions. In such cases, to insure a fairly accurate determination of the food intake, the cage was placed over a crystallizing dish of such size as to fit under it tightly. This prevented the inroads of any wild mice. To insure a good separation of food waste and urine, a piece of heavy filter paper about 4 inches square was placed immediately under the food crucible in the crystallizing dish. With these arrangements it was rare that a good determination of the food intake could not be made, though only at the expense of much time and patience. The food was weighed out once a day, at which time the uneaten food from the preceding day was determined. The mice were weighed once a day in the amino-acid experiments, and once every other day in the experiments with other rations.

Water was supplied to the mice from an inverted bottle (attached to the removable top of the cage) provided with a bent delivery tube protruding a few inches into the cage. This method is very satisfactory, if care is taken to insure that the delivery tubes are always full of water. This depends primarily on the bore of the tube, the angle at which it is bent, and the size of the opening at the end of the tube, which is necessarily constricted somewhat to prevent undue loss of water. Distilled water only was used.

The mice were kept in light and dry quarters. During the autumn months when no artificial heat was available, the temperature occasionally fell to 65° or even 60° F., but later after the installation of a radiator, the temperature always remained above 70° and was generally maintained between 75° and 80°. In the hottest days of summer the temperature often rose above 90° F., but at such times conditions were alleviated somewhat by the use of an electric fan.

The cages were cleaned out once a day generally, and in all cases at least once every other day. They were sterilized whenever such measures were required (generally once in 1 or 2 weeks) by passage through a hot solution of strong alkali and thorough rinsing with water.

The amino-acids used in the feeding experiments were mostly prepared from protein material. The tyrosine, cystine, glutamic

acid, lysine, histidine, and arginine were prepared in the usual way from various proteins after complete acid hydrolysis. The tryptophane was prepared from pancreatine digests of casein by the method of Hopkins and Cole. The valine was synthesized, and the aspartic acid, asparagine, leucine, alanine, glycocoll, isoleucine, and phenylalanine were bought on the market and were in most cases Kahlbaum products. The *l*-proline used in some of the rations was an impure product obtained by alcohol extraction of protein hydrolysates with acid and subsequent isolation of the copper salt. Oxyproline and serine were not available for any of this work.

Most of the amino-acids prepared in the laboratory were not of tested purity. The tyrosine and cystine used were found to contain very close to the theoretical percentage of nitrogen and one tryptophane preparation was likewise found to be a very good product as judged by this criterion.

Basal Rations for Maintenance.—In view of the fact that growth probably sets a higher standard as regards amino-acid requirements than does simple maintenance, the logical point of attack in this investigation appeared to be the amino-acid requirements for maintenance rather than for growth. Another argument in favor of this procedure was that adult mice, which would be used in maintenance experiments, could probably withstand the digestive disturbances anticipated in the feeding of amino-acid rations better than immature animals.

It was therefore necessary to decide upon a suitable basal ration for maintenance made up of food substances containing minimal amounts of nitrogen. The rations used by McCollum and Davis, consisting of dextrin, lactose, purified butter fat, agar-agar, and a synthetic salt mixture, besides protein, for which mixtures of amino-acids could be substituted in our experiments, seemed ideal for the purpose. Unfortunately we could not secure long continued maintenance of mice with such a ration, even when casein was used to cover the nitrogen requirements, the trouble seeming to rest with the salt mixture. The ration of Osborne and Mendel, consisting of protein 18 per cent, "protein-free milk" 28 per cent, starch 26 per cent, lard 10 per cent, and purified butter fat 18 per cent, was found to be capable of maintaining

mice for long periods of time, thus confirming the results of Wheeler.[18, 26]

The main objection to such a ration in an experiment of this nature is the fact that the "protein-free milk" as prepared according to the directions of Osborne and Mendel always contains protein nitrogen. In attempting to obviate this difficulty we have slightly modified the method of preparing this product.[27] After precipitating the casein from milk by a slight excess of HCl, filtering off the coagulum, boiling for ½ minute to coagulate the lactalbumin, and filtering off this precipitate, the solution was carefully neutralized to litmus with NaOH and again filtered, the filtrate being then evaporated to a thick paste on the water bath at 70°C. and dried in a current of warm air. The last filtration, which constitutes the modification introduced, removes about 0.1 per cent of nitrogen, presumably protein nitrogen, figured on the basis of the dried product, or one-seventh of the total nitrogen of the first filtrate. Some of the mineral salts are also removed, though the nutritive value of the preparation for maintenance does not seem to be impaired at all if it constitutes as much as 28 per cent of the ration. This modified preparation of "protein-free milk" was used in many of the feeding experiments reported below.

Feeding Experiments with Mixtures of Amino-Acids.—In the first attempts to cover the nitrogenous requirements of mice by defi-

[26] While the Osborne and Mendel casein and "protein-free milk" ration appears to be satisfactory for the maintenance of weight and health of white mice, it does not appear to be satisfactory for the production of offspring. Comparatively few cases of pregnancy were observed with mice on this ration. Where pregnancy occurred birth was generally premature, apparently, and the young were generally eaten by the mother within 24 or 48 hours. In the course of over a year's experience only three young (out of a litter of five) were successfully raised until weaning, on this ration, and in these cases growth during the nursing period was noticeably slower than normal.

[27] Mitchell, H. H., and Nelson, R. A., *J. Biol. Chem.*, 1915, xxiii, 459. It might be said that practically all of the investigation work reported in this paper was completed before the trichloroacetic acid method of preparing protein-free milk was elaborated. This method, while probably better suited to the purposes of this work than any other, was not used in the preparation of any of the rations tested.

nite mixtures of amino-acids, the Osborne-Mendel ration was modified simply by the substitution of the 18 per cent casein by 18 per cent of definite mixtures of amino-acids, containing fifteen or fewer of the seventeen amino-acids commonly found in proteins, the two amino-acids lacking being serine and oxyproline. The proportions of the individual amino-acids included in these mixtures were more or less arbitrarily chosen, since there is little information at hand that would logically furnish a presumption in favor of any one mixture as opposed to others. In preparing these (as well as other mixtures tested) some consideration was given to the available analyses of pure proteins known to be complete for maintenance and growth; more consideration was necessarily paid to the stock of amino-acids on hand.

The results of the feeding experiments with 18 per cent of amino-acids were not successful and will not be given in detail. In all cases a decline in weight began as soon as the amino-acid rations were given, and in all cases the food consumed per day was light, especially as the experiment continued, so that it cannot be said that the failure to maintain weight was due to an incomplete amino-acid intake any more than to an incomplete energy intake. In several cases the mice on these amino-acid rations died within 2 weeks, and in other cases after longer periods. Four of the mice that died showed symptoms of severe diarrhea. The other six mice that died on the amino-acid rations showed no outward symptoms of digestive disturbance. No postmortem examinations of these mice were made.

The most successful of these preliminary experiments were run on two male and two female mice. The former two mice weighed from 20 to 21 gm. at the beginning of the experiment and were kept on rations containing mixtures of the 15 amino-acids available, for 27 days. At this time they had lost 37 and 45 per cent of their original weight. The two female mice weighed from 18 to 18.5 gm. initially and were kept on an amino-acid ration for 28 days, at which time they had lost 37 and 43 per cent of their weight. The condition of these four mice at the end of 27 and 28 days was obviously poor. Their hair was rough, emaciation was extreme, and they were so weak that they could barely use their hind legs. Upon changing to a ration containing 18 per cent of casein, recovery of the original weight occurred slowly in three

cases. In one case recovery was not complete, though the health and condition of the animal was apparently restored.

At this stage of the investigation the publications of Osborne and Mendel[11] and of McCollum and Davis[28] on minimum protein requirements appeared, in which it was shown that the protein requirements for the maintenance of rats may be covered by rations containing as low as 3 or even 2 per cent of protein. A plan for further work with the amino-acid rations immediately suggested itself. By lowering the percentage of amino-acids in the ration it was hoped that the food intake of the mice could be raised to normal and thus assure a fair test of the amino-acid mixtures, provided that the diminished consumption of the 18 per cent amino-acid rations was due simply to some disagreeable flavor imparted to the rations by the amino-acids contained in them.

This plan was first tested with a ration containing 8.1 per cent of a mixture of six amino-acids:

	per cent
Tyrosine	1.0
Cystine	1.0
Glutamic acid	4.0
Tryptophane	0.6
Histidine	0.5
Arginine	1.0

This ration was given to a male mouse with an initial weight of 21.4 gm. For the first 9 days the food intake was adequate, apparently, in covering the energy requirements, being from 2 to 3 gm. per day. The body weight decreased steadily, however, probably due to an inadequate amino-acid intake. On the 9th day the mouse weighed only 15.6 gm. From the 9th through the 50th day of the experiment the amino-acid ration was alternated in an irregular fashion with a non-nitrogenous ration, containing neither protein nor amino-acids except in so far as these are contained in "protein-free milk."

The introduction of the non-nitrogenous ration exerted a decidedly favorable influence on the body weight, which showed no tendency to decrease during the next 16 days of observation—on the 25th day of observation the weight of the mouse was 15.2

[28] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 415.

gm. However, from here on, a slow decline ensued. During the period of mixed feeding the food consumption was much better on the non-nitrogenous than on the amino-acid rations, and almost invariably a day's feeding of the former when immediately preceded by a day's feeding of the latter ration produced a more or less clearly marked increase in body weight. For 50 days this mouse was kept alive on rations containing no appreciable amount of protein. Towards the last of the test the animal was in very poor condition and weighed 11.4 gm. on the 50th day. An attempt at recuperation on rations containing small amounts of casein (2 to 3 per cent) supplemented with cystine and tryptophane failed, and the mouse died on the 58th day of the experiment at a weight of 10.2 gm. The total loss in weight amounted to about 52 per cent of the initial weight.

A somewhat similar experiment to that just described was undertaken with a male and female mouse each weighing initially about 23 gm. For the first 9 days of the experiment these mice were put upon a non-nitrogenous ration and they declined steadily and rapidly in weight. From the 9th to the 40th days non-nitrogenous and amino-acid rations were fed alternately, as in the preceding experiment. The decline in weight still continued, but at a distinctly more moderate rate, though the food consumption was not noticeably increased, decreasing slowly, in fact, both absolutely and per 100 gm. of body weight.

These two experiments showed that the body weight curves of mice on amino-acid rations could be favorably affected by interposing non-nitrogenous rations alternately with the test rations. This favorable effect may be due simply to the fact that the former rations were consumed in larger quantities than the latter so that the energy requirements could be covered, or at least more nearly covered, than on amino-acid rations entirely. While this régime at the same time lowers the intake of amino-acids, it might still be consistent with nitrogen equilibrium in view of the known low protein requirements for maintenance of rats, and presumably also of mice. Therefore a series of feeding experiments were planned to test the value of various amino-acid mixtures which were included in percentages ranging from 2.5 to 9 in rations containing 28 per cent of "protein-free milk" (in some cases the modified preparation described above), 26 per cent starch, 10 per cent

lard, 18 per cent butter fat, and sufficient sucrose to make the ration up to 100 per cent. These amino-acid rations were fed alternately with a non-nitrogenous ration consisting of 10 per cent sucrose, 34 per cent starch, 28 per cent "protein-free milk" (in most cases the modified preparation), 10 per cent lard, and 18 per cent butter fat. A few of the more successful of these experiments, the results of which are represented graphically in Charts I to V, will now be considered.

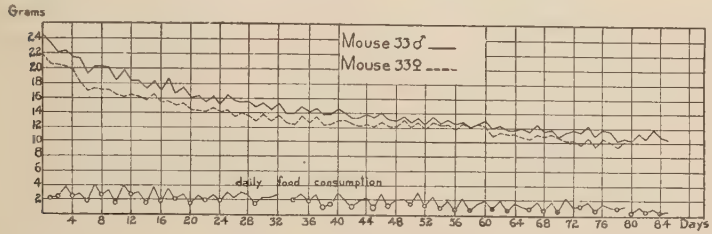

Chart I. The upper two curves represent the changes in body weight of Mice 33 ♂ and 33 ♀. The circled points on the lower curve, representing the daily food consumption of the mice, indicate the days on which amino-acid rations were consumed. On the other days practically non-nitrogenous rations were given. For the period up to and including the 59th day the amino-acid ration used contained: 0.20 per cent histidine, 0.25 per cent glycocoll, alanine, valine, isoleucine, phenylalanine, proline, and aspartic acid, 0.30 per cent tryptophane, 0.37 per cent cystine, 0.40 per cent arginine and lysine, 0.45 per cent asparagine, 0.50 per cent leucine and tyrosine, and 0.75 per cent glutamic acid—a total of 5.62 per cent of amino-acids. This ration contained the most complete amino-acid mixture used in all the experiments reported; *i.e.*, fifteen acids. Of the amino-acids commonly occurring in proteins only serine and oxyproline are not represented. From the 60th to the 76th days inclusive the amino-acid ration used contained: 0.25 per cent isoleucine, cystine, tryptophane, arginine, histidine, valine, aspartic acid, and phenylalanine, 0.50 per cent tyrosine and leucine, and 1.00 per cent glutamic acid—a total of eleven amino-acids, making up 4.00 per cent of the ration. From the 77th day to the end of the experiment the amino-acid ration used contained ten amino-acids: 0.25 per cent cystine, tryptophane, valine, histidine, and phenylalanine, 0.50 per cent tyrosine, leucine, arginine, and asparagine, and 0.75 per cent glutamic acid—a total of 4.00 per cent.

In Chart I are recorded the results of a feeding experiment on two mice that were fed alternately, as indicated by the curve at the bottom of the figure representing the daily food consumption, a non-nitrogenous ration and an amino-acid ration containing

5.62 per cent of a mixture of fifteen amino-acids—the most complete amino-acid mixture tested in this investigation. Of the amino-acids commonly reported as occurring in proteins, this mixture lacked only serine and oxyproline. This system of feeding was continued for 59 days, during which period Mouse 33 ♂ decreased very gradually in weight from 24.4 to 12.6 gm., a total loss of over 48 per cent of the original weight. In the same period Mouse 33 ♀ decreased in weight for about 35 days from 21.7 gm. to a level of 12 to 13 gm. This level was maintained until the amino-acid ration was changed on the 59th day of the experiment; that is, for a period of over 3 weeks the weight of this mouse was maintained, though it was evident from its appearance that it was not in a good nutritive condition. On the 59th day of the experiment the amino-acid ration was changed to one containing 4 per cent of a mixture of only eleven amino-acids; *i.e.*, lacking glycocoll, alanine, proline, and lysine, as well as serine and oxyproline. The effect of this change was well marked on Mouse 33 ♀, which declined in weight continuously until its death on the 80th day of the experiment, at a weight of 10.1 gm. The total loss in weight of this mouse amounted to 53 per cent of the initial weight. However, the change in ration had no perceptible effect on Mouse 33 ♂, which continued its decline in weight at a uniform rate until its death on the 85th day of the experiment. The final weight of this mouse was 10.5 gm. and the total loss in weight during the entire experiment, 57 per cent of the initial weight. On the 77th day of this experiment the amino-acid ration was changed to one containing only ten amino-acids, as indicated on the chart.

A second feeding experiment, illustrated by Chart II, was undertaken with the second amino-acid ration used in the preceding experiment; *i.e.*, the ration containing 4 per cent of a mixture of eleven amino-acids. On this ration, alternated with the non-nitrogenous ration, Mouse 32 ♂ decreased in weight almost continuously (aside from the fluctuations caused by the alternation of rations) from an initial weight of 21.5 gm. to 15.5 gm. on the 35th day. At this point the weight increased rapidly for 4 or 5 days and then decreased suddenly. The mouse was found dead in the cage on the 45th day of observation, and was partially eaten by Mouse 32 ♀. At the time of this sudden increase in weight

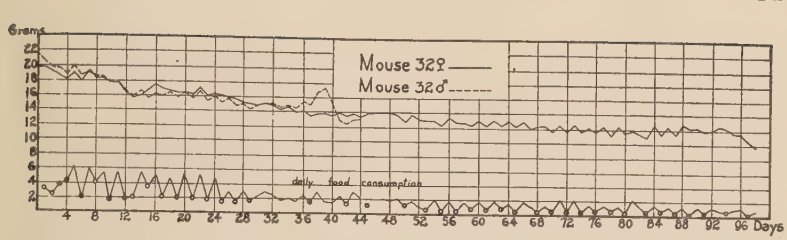

CHART II. The data for Mice 32 ♂ and 32 ♀ are presented in the above curves in a manner similar to the data in Chart I. For the period up to and including the 83rd day the amino-acid ration used contained: 0.25 per cent isoleucine, cystine, tryptophane, arginine, histidine, valine, aspartic acid, and phenylalanine, 0.50 per cent leucine and tyrosine, and 1.00 per cent glutamic acid—a total of 4.00 per cent of eleven amino-acids. From the 83rd day to the end of the experiment the amino-acid ration given contained: 0.25 per cent cystine, tryptophane, valine, histidine, and phenylalanine, 0.50 per cent tyrosine, leucine, arginine, and asparagine, and 0.75 per cent glutamic acid—a total of 4.00 per cent of a mixture of ten amino-acids.

the mouse had a puffy unhealthy appearance, probably due to an accumulation of water in the tissues. During the 45 days of observation this mouse lost 39 per cent of its weight. Mouse 32 ♀ decreased in weight regularly, but at a slower and slower rate; from about the 60th to the 94th day the weight was practically constant as is indicated by the weights tabulated below:

Weight of Mouse 32 ♀.

Day of test.	*gm.*
55	12.4
60	13.0
65	12.4
70	11.8
75	12.2
80	11.7
85	11.5
90	12.3
95	11.7

On the 83rd day of the experiment the amino-acid ration was changed to one containing 4 per cent of a mixture of ten acids, the lack of isoleucine constituting the main difference between this and the preceding ration. On the 98th day of the experiment the mouse died at a weight of 9.8 gm., the total loss of weight during the entire experiment amounting to 50.5 per cent of the

initial weight. The mouse was active and appeared to possess a normal appetite until the last 5 or 6 days, when it became noticeably weak, assumed a very anemic appearance, and contracted some infection in one eye.

A third mouse, No. 50 ♂, was put on the amino-acid ration used for the first 82 days of the experiment just described, fed, as usual, alternately with a non-nitrogenous ration. For 12 days immediately preceding this system of feeding, the mouse had been on an amino-acid ration containing only 2.5 per cent of a mixture of eight amino-acids. Throughout the period of feeding on both of these rations the weight of the mouse declined slowly but steadily. The rate of decline was not apparently affected by the change in feeding instituted on the 12th day of observation. On the 57th day of the experiment the mouse died at a weight of 12.5 gm., having lost 44 per cent of its original weight (22.5 gm.).

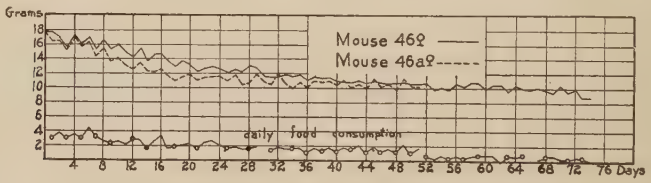

Chart III. The data for Mice 46 ♀ and 46 a ♀ are presented in the above curves in a similar manner to the data in Chart I. The amino-acid ration used throughout the experiment contained 0.25 per cent cystine, tryptophane, valine, histidine, and phenylalanine, 0.50 per cent tyrosine, leucine, arginine, and asparagine, and 0.75 per cent glutamic acid—a total of ten amino-acids, accounting for 4.00 per cent of the ration.

In Chart III are given the results of an experiment on two mice to test the nutritive value of a mixture of ten amino-acids, constituting 4 per cent of a ration that was fed alternately with a non-nitrogenous ration. The proportions in which each amino-acid entered into the former ration are as follows:

	per cent		*per cent*
Glutamic acid	0.75	Cystine	0.25
Tyrosine	0.50	Tryptophane	0.25
Leucine	0.50	Valine	0.25
Arginine	0.50	Histidine	0.25
Asparagine	0.50	Phenylalanine	0.25
		Total	4.00

With this system of feeding Mouse 46 a ♀ decreased in weight from 17.7 gm. to a level of 10 to 11 gm. in a matter of about 30 to 35 days. This level was maintained until the 51st day of the experiment when the mouse was found dead at a weight of 10.2 gm.; that is, for a period of about 3 weeks. Mouse 46 ♀ decreased from an initial weight of 17.7 gm. to a level of 10 to 11 gm. in about 40 days. This level was maintained with insignificant changes until about the 70th day of observation, at which time a rapid falling off in weight occurred until the death of the animal on the 74th day at a weight of 8.9 gm. The following weights of this mouse illustrate the condition of practical maintenance existing from the 40th to the 70th days:

Weight of Mouse 46 ♀.

Day of test.	*gm.*
40	11.0
45	11.0
50	10.6
55	9.7
60	10.0
65	10.0
70	10.2

In this experiment Mouse 46 a ♀ lost 42 per cent of its initial weight, and Mouse 46 ♀ lost almost exactly 50 per cent.

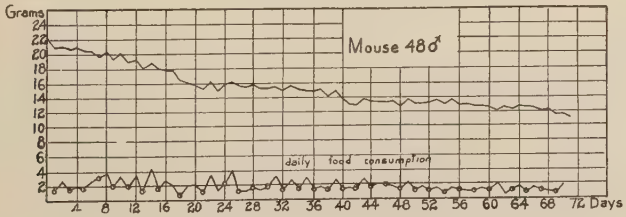

CHART IV. The data for Mouse 48 ♂ are presented in the above curves in a similar manner to the data in Chart I. The amino-acid ration used throughout this experiment contained 0.25 per cent cystine, valine, and histidine, 0.30 per cent tryptophane, 0.45 per cent arginine, 0.50 per cent leucine, asparagine, and phenylalanine, and 1.00 per cent glutamic acid—a total of 4.00 per cent of nine amino-acids. It is to be noted especially that the ration contains no tyrosine.

In the experiments thus far considered, tryptophane, tyrosine, and cystine, the amino-acids known to be indispensable for the maintenance of life, have in all cases been incorporated in the amino-acid mixtures tested. In Chart IV are given the results of a feeding experiment with an amino-acid ration containing 0.25 per cent of cystine, valine, and histidine, 0.30 per cent of tryptophane, 0.45 per cent of arginine, 0.50 per cent of leucine, asparagine, and phenylalanine, and 1.00 per cent of glutamic acid—a total of 4.00 per cent of nine amino-acids, among which tyrosine was not included. On this ration, alternated with the non-nitrogenous ration, Mouse 48 ♂ decreased in weight slowly but continuously from 22.1 gm. to a level of 13.0 to 13.5 gm. in the course of 40 days. This level was maintained for a period of 15 to 20 days, as the following figures show:

Weight of Mouse 48 ♂.

Day of test.	*gm.*
41	13.0
45	13.2
50	13.1
55	13.5
60	12.6

From this time on, the weight decreasd slowly until the death of the animal on the 72nd day. The final weight was 11.0 gm. and the total loss in weight amounted to 50 per cent of the initial weight.

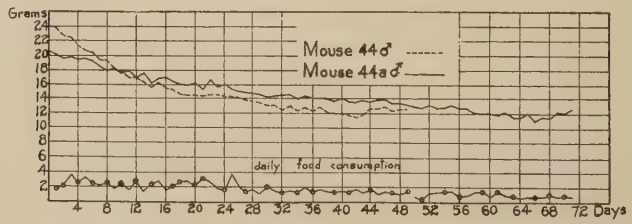

Chart V. The data for Mice 44 ♂ and 44a ♂ are presented in the above curves in a similar manner to the data in Chart I. The amino-acid ration used throughout this experiment contained 0.25 per cent cystine, valine, and histidine, 0.30 per cent tryptophane, 0.45 per cent arginine, 0.75 per cent leucine and asparagine, and 1.00 per cent glutamic acid—a total of 4.00 per cent of amino-acids. It is to be noted that this ration contained only eight amino-acids and, in particular, is lacking in tyrosine and phenylalanine.

A still less complete amino-acid mixture than the above was tested in an experiment the results of which are given in Chart V. The amino-acid ration used in this experiment contained 0.25 per cent of cystine, valine, and histidine, 0.30 per cent of tryptophane, 0.45 per cent of arginine, 0.75 per cent of leucine and asparagine, and 1.00 per cent of glutamic acid—a total of 4.00 per cent of amino-acids. It is to be noted that this mixture contains only eight amino-acids, and, in particular, lacks both tyrosine and phenylalanine. On this ration, fed alternately with the non-nitrogenous ration, Mouse 44 a ♂ declined comparatively rapidly from an initial weight of 24.0 gm. to a weight of 11.5 gm. on the 42nd day of feeding. A slight increase in weight preceded the death of this mouse on the 49th day of the experiment at a final weight of 12.6 gm., the total loss in weight amounting to 47 per cent of the initial weight. The decline of Mouse 44 ♂ was much more gradual, though continuous, and death ensued on the 72nd day of observation after a loss of 39 per cent of its initial weight.

The length of time that mice could be kept alive on mixtures of only a few amino-acids not including tyrosine or even tyrosine and phenylalanine, seemed remarkable. The withdrawal of tryptophane, however, appeared to exert a distinctly more detrimental effect. Three mice were fed alternately a non-nitrogenous ration and an amino-acid ration containing 0.25 per cent of cystine, valine, histidine, and phenylalanine, 0.45 per cent of arginine, 0.50 per cent of tyrosine, leucine, and asparagine, and 1.05 per cent of glutamic acid—a total of 4.00 per cent of nine amino-acids, excluding tryptophane. All three mice decreased continuously in weight. One died on the 33rd day after a loss of 32 per cent of its initial weight (22.8 gm.); another died on the 39th day of feeding, having lost 48 per cent of its original weight (20.1 gm.); the third mouse died on the 44th day of observation, the total loss in weight amounting to 44 per cent of its initial weight (22.0 gm.).

In studying the series of experiments just described on the nutritive value of different mixtures of amino-acids, one is struck by the fact that, aside from the mixture from which tryptophane was excluded, no clearly marked differences in nutritive value (with possibly one exception, mentioned above) are revealed as judged by the rate of decline in body weight or by the compara-

tive longevity of the experimental animals. The different mixtures were often strikingly different in general make-up, containing from eight to fifteen amino-acids in varying proportions. This general similarity in body weight variations is the more surprising in view of experiments on the feeding of isolated proteins inadequate for maintenance due to deficiencies in amino-acid content. These studies show that the different proteins exhibit more or less characteristic effects on the body weight of rats and mice. For example, phaseolin leads to a slow decline in body weight, zein to a more rapid decline, and gelatin to a still more rapid decline. As regards the last two proteins, Wheeler has shown[29] in the case of albino mice that with zein one-third of the body weight was lost in 25 days, while with gelatin one-third was lost in 12 days. It is true that the absence of data of the food intake precludes an analysis of the exact significance of such results. Furthermore, Osborne and Mendel[30] have shown that rations containing 2 per cent of proteins qualitatively adequate for maintenance have rather distinct characteristic effects on the rate of decline of rats, those containing edestin, for example, more nearly maintaining rats than those containing gliadin or glycinine. Thus, it is natural to expect that amino-acid mixtures characterized by qualitatively different types of deficiencies should exhibit distinct characteristic effects, and that such effects were not produced in the experiments reported here is of interest.

In speculating on the causes of the decreases in body weight obtained with all of the amino-acid rations, it must be conceded that probably in no case was enough of the amino-acid mixture consumed to really constitute a fair test of its adequacy. Although the favorable effects of feeding the amino-acid rations used in these experiments, not continuously, but alternately with non-nitrogeneous rations, seem evident, it seems to be a fact that this alternation of the two types of ration, while in most cases it assured a sufficient calorie intake, operated against the attainment of a sufficient amino-acid intake, unless this is much smaller than has hitherto been supposed.

It was surprising how the experimental animals could differentiate between rations containing no added amino-acids, and

[29] Wheeler, *J. Exp. Zool.*, 1913, xv, 215.

[30] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 377.

those containing even very small concentrations of amino-acids. Inspection of Charts I to V is sufficient to show that the non-nitrogenous rations were in general consumed to a much greater extent than the rations containing 6 or 4 per cent of amino-acids. Even in the case of rations containing 2.5 per cent of amino-acids the same marked difference in intake has been observed. It seems impossible that this distinction could have been made on the basis of a difference in taste.

In order to be in a position to appreciate fully the above experiments, feeding tests were made on seven mice using the non-nitrogenous ration only, containing 28 per cent of the modified "protein-free milk." In all of these tests the mice decreased steadily in weight until death, though the periods of survival were different, as Table I shows.

TABLE I.

*Records of Mice on Non-Nitrogenous Ration.**

Mouse.	Period of survival.	Initial weight.	Final weight.	Total loss in weight in per cent of initial weight.
	days	*gm.*	*gm.*	*per cent*
48 ♀	63	19.9	11.1	44
43 ♂	38	26.1	11.3	57
43 ♀	13	20.0	11.8	41
80 ♂	44	27.6	14.2	49
80a ♂	30	24.4	15.9	35
81 ♂	40	24.9	11.9	52
81a ♂	36	22.6	12.0	47

* This ration consisted of sucrose 10 per cent, starch 34 per cent, modified protein-free milk 28 per cent, lard 10 per cent, and butter fat 18 per cent.

It is evident from the results reported in this table that the periods of survival of the mice on the non-nitrogenous ration were distinctly less than the periods of survival of the mice in the more successful feeding experiments with the amino-acid and non-nitrogenous rations fed alternately. Aside from the exceptional behavior of Mouse 48 ♀, the survival periods of the mice on the non-nitrogenous rations are rather strikingly similar to the survival periods of the mice on the amino-acid ration not containing tryptophane; *i.e.*, 33, 39, and 44 days.

That the longer survival of mice receiving amino-acids in their food as compared with mice receiving no amino-acids was not due to a more liberal consumption of food is shown in Table II, in which the average daily consumption of food per 100 gm. of body weight for successive 10 day periods is given (1) for the amino-acid experiments graphically represented in Charts I to V, and (2) for the feeding experiments with the non-nitrogenous ration just described. In the former case, the averages include both the amino-acid and non-nitrogenous ration, which were of approximately equal energy value.

TABLE II.

The Average Daily Food Consumption per 100 Gm. of Body Weight for Successive 10 Day Periods in Feeding Experiments (1) with Amino-Acid Rations, (2) with the Non-Nitrogenous Ration Only, and (3) with an Asparagine Ration. Results Expressed in Gm.

10 day period.	Amino-acid experiments.					Non-nitrogenous experiments.				Asparagine experiments.	
	33 ♂ and 33 ♀	32 ♂ and 32 ♀	46 ♀ and 46a ♀	48 ♂	44 ♂ and 44a ♂	48 ♀	43 ♂ and 43 ♀	80 ♂ and 80a ♂	81 ♂ and 81a ♂	82 ♂	84 ♀
1	6.5	9.9	9.5	11.5	6.1	10.6	9.7	14.6	16.9	9.2	8.3
2	7.9	11.0	8.8	12.2	6.9	11.6		15.6	15.8	8.5	9.1
3	8.1	8.3	8.0	12.2	7.3	8.1	9.3	13.5	14.2	8.9	5.4
4	8.1	7.0	7.1	13.5	5.6	9.5		11.1		7.6	4.7
5	6.9	9.0	7.6	13.5	5.0	7.3				9.0	
6	6.6	9.6	5.9	9.8	8.5	8.6					
7	5.2	8.0	5.7	10.5	7.2						
8	5.8	9.3									
9		8.9									
10		5.3*									

* Average for 8 days only.

In the last two columns of Table II are given the average daily food consumption for two mice on a ration containing 10 per cent asparagine, 10 per cent sucrose, 24 per cent starch, 28 per cent modified protein-free milk, 10 per cent lard, and 18 per cent butter fat. On this ration Mouse 84 ♀ lived for 44 days, losing 56 per cent of its original weight, while Mouse 82 ♂ lived for 57 days, with a total loss of 50 per cent of the initial weight.

It can hardly be said that the asparagine ration was more successful than the non-nitrogenous ration as regards the period

of survival. However, it seems fairly conclusive that the amino-acid experiments as a whole were much more successful in this regard than the experiments involving the feeding of the non-nitrogenous ration, and that this difference in nutritive efficiency cannot be explained by a more liberal consumption of food in the amino-acid experiments; in fact, the reverse was more truly the case. It seems, therefore, that many of the amino-acid mixtures fed were utilized by the mice in some way in preserving intact the normal functioning of the tissues, possibly, as has been suggested, by furnishing the necessary precursors for the elaboration of the active constituents of the internal secretions, even though such mixtures were not sufficiently complete to make possible the synthesis of protein. These results, therefore, confirm the belief, rapidly gaining ground, that some if not all of the amino-acids occurring in combination in the protein molecule have specific functions in the animal economy aside from the function of serving simply as structural units in the elaboration of the body proteins.

According to the generally accepted theories of protein metabolism, a protein inadequate for maintenance, such as zein or gelatin, that is, a protein totally deficient in one or more of the amino-acids that are necessary for the construction of body protein because it cannot be synthesized by the body, would be of no use in metabolism except as a source of energy. However, McCollum[31] has shown that the pig

"can utilize the nitrogen of zein very efficiently for repair of the losses due to endogenous or tissue metabolism. The average utilization of zein nitrogen was about 80 per cent, for gelatin 50 to 60 per cent. No evidence was obtained of the formation of additional body tissue from zein, even when the latter was fed in great excess over the maintenance needs of the animals."

In other words, assuming a constant endogenous metabolism, these percentages of the nitrogen of zein and gelatin were retained by the animals and apparently utilized for some purpose other than the construction of protein tissue. Similarly, Henriques and Hansen[32] have shown that the protamine clupein, consisting

[31] McCollum, E. V., *Wisconsin Agric. Exp. Station, Research Bull. 21*, 1912.
[32] Henriques and Hansen, *Z. physiol. Chem.*, 1906, xlix, 113.

of arginine to the extent of about 82 to 83 per cent, was inadequate for maintenance of the white rat, but that its nitrogen was, nevertheless, utilized to some extent in metabolism. Recently Abderhalden[4] has reported experimental results tending to show that, if mice are subjected to protein starvation, while receiving plenty of the non-nitrogenous nutrients, and if in an immediately following period, alanine or glycocoll is added to the basal ration, these amino-acids effect a reduction of the nitrogen loss of the first period, indicating a specific utilization of these nitrogenous nutrients, even when fed singly.

Similarly, the results of the experiments of this paper show that mice survive longer on the feeding of incomplete mixtures of amino-acids added to a basal ration of non-nitrogenous nutrients, than on the feeding of the non-nitrogenous nutrients alone. The most successful experiments of the former type extended over periods 75 to 100 per cent longer than the more representative experiments of the latter type. It appears, therefore, that the requirements of the animal organism for amino-acids resemble its requirement for inorganic ions, in that for the maintenance of life over long periods of time a complete assortment both of amino-acids and mineral elements is essential, while at the same time each essential amino-acid and mineral element has a specific function in metabolism. Thus, incomplete mixtures of either amino-acids or mineral elements will partially cover the body's requirements in the proportion of their completeness, though the elaboration of new tissue, and probably the efficient repair of old tissue, will be impossible in the absence of only one of the essential constituents.

SUMMARY AND CONCLUSIONS.

1. Experiments are reported in this paper in which mice have been kept alive for 70 to 98 days by feeding alternately (1) rations containing 4 to 6 per cent of various mixtures of isolated amino-acids, 6 to 4 per cent sucrose, 34 per cent starch, 28 per cent protein-free milk (prepared either according to the procedure of Osborne and Mendel or to a modification of this procedure described in the text), 10 per cent lard, and 18 per cent butter

fat, and (2) a ration containing 10 per cent sucrose, with other constitutents in the same proportion as in the first mentioned rations. In many of these experiments periods of 15 to 35 days' duration have been observed in which the mice practically maintained their weight.

2. The alternate feeding of an amino-acid ration and a non-nitrogenous ration (except for the nitrogen present in the protein-free milk) induced a better total consumption of food than feeding with an amino-acid ration alone, and in all other respects led to more successful results. However, it is probable that in no case was the amino-acid intake sufficiently large to assure a fair test of its adequacy.

3. Amino-acid rations containing no added tyrosine, or no added tyrosine and phenylalanine, did not give appreciably different results from rations containing these amino-acids. However, if tryptophane was absent from an amino-acid ration, the period of survival of mice fed this ration alternately with the non-nitrogenous ration was noticeably shorter than the periods of survival of mice kept on rations containing added tryptophane.

4. Mice could be kept for much longer periods of time on rations containing mixtures of amino-acids, including tryptophane, fed alternately with the non-nitrogenous basal ration, than when fed the basal ration alone. Furthermore, this difference in survival cannot be accounted for by a difference in energy intake. This fact has been interpreted as meaning that at least some of the amino-acids have specific functions in metabolism aside from that of serving simply as material for the synthesis of body protein. Other evidence from the literature is cited in support of this view, which is substantially the same as that recently and tentatively put forward by Osborne and Mendel.

In conclusion I wish to acknowledge the general supervision of this work by Professor H. S. Grindley, and the aid which his suggestions have contributed. The assistance of Mr. Anton Prasil and Mr. R. A. Nelson in the work of preparing and synthesizing the amino-acids used, is also appreciated.

BIOGRAPHICAL SKETCH

The writer was born at Evanston, Illinois, January 22, 1886, and received his elementary education in the public schools of this city and of Chicago, Illinois. After three and a half years attendance at the Hyde Park and the Austin High Schools of Chicago, he entered the University of Illinois in the fall of 1904. He received the degree of Bachelor of Arts in the general science course in June, 1909. After three years in the graduate school of the University of Illinois he received the degree of Master of Science in Chemistry in 1913.

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The writer has published the following papers:

1. A Study of the Food Requirements of a Group of Twenty-Four Men (with H. S. Grindley). Presented before the American Chemical Society, July 1, 1909. Abstract published in Science, Vol. 30, p. 241. 1909.

2. On the Metabolism Experiment as a Statistical Problem (with H. L. Rietz), Journal of Biological Chemistry, Vol. 8, pp. 297-326. October 1910.

3. The Element of Uncertainty in the Interpretation of Feeding Experiments (with H. S. Grindley). Illinois Agricultural Experiment Station, Bulletin 165. 117 pp. July 1913.

4. Does a Low Protein Diet Produce Racial Inferiority? Science, Vol. 38, pp. 82-83. 1913.

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